

University of Groningen

Impact of the enzyme flexibility on the enzyme enantio- selectivity in organic media towards specific and non-specific substrates

Broos, J.

Published in:
Biocatalysis and Biotransformation

DOI:
[10.1080/10242420290004938](https://doi.org/10.1080/10242420290004938)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2002

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Broos, J. (2002). Impact of the enzyme flexibility on the enzyme enantio- selectivity in organic media towards specific and non-specific substrates. *Biocatalysis and Biotransformation*, 20(4), 291 - 295.
<https://doi.org/10.1080/10242420290004938>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Short communication

Impact of the Enzyme Flexibility on the Enzyme Enantioselectivity in Organic Media Towards Specific and Non-specific Substrates

JAAP BROOS*

Department of Biochemistry and Groningen Biomolecular Science and Biotechnology Institute (GBB), University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

(Received 14 June 2001; Revised 5 October 2001)

A rationale is presented as to why the enantioselectivity of enzymes in dry organic media towards specific substrates is increased when the enzyme flexibility is increased. This is outlined for serine proteases towards N-acetyl-amino acid esters. The reasons why this relationship does not hold in the case of non-specific substrates are discussed.

Keywords: Enzyme enantioselectivity; Enzyme flexibility; Organic media; Specific substrates

INTRODUCTION

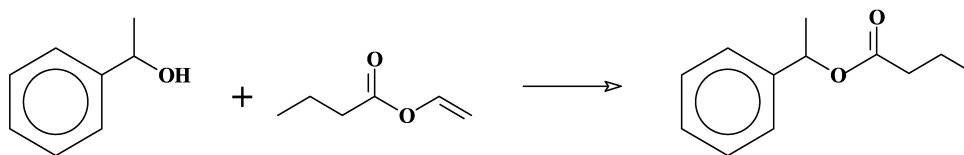
The enantioselectivity of enzymes can be completely different when suspended in a dry organic medium compared from the enantioselectivity exhibited in water. Rationalization of the enzyme enantioselectivity under non-aqueous conditions is one of the most challenging topics within the field of non-aqueous enzymology. Several physicochemical properties of the organic medium like solvent hydrophobicity (log P), dielectric constant, dipole moment, and solvent size have been proposed to effect enzyme enantioselectivity. Relationships between these parameters and enzyme enantioselectivity have been reported for numerous enzymes and reaction types (for reviews see Carrea *et al.*, 1995; Koskinen and Klivanov, 1996; Halling, 2000). A

uniform model to predict the enzyme enantioselectivity under non-aqueous conditions is not available yet. An important difference between aqueous and non-aqueous enzymology is the restricted flexibility of an enzyme at low water activities (Rupley and Careri, 1991). The impact of enzyme flexibility on enzyme enantioselectivity was addressed by Broos *et al.* several years ago (Broos *et al.*, 1995c). For the reaction system investigated, it was found that an increase in enzyme flexibility correlated with a higher enzyme enantioselectivity. In a recent contribution, Rariy and Klivanov challenged this conclusion (Rariy and Klivanov, 2000). In their view, inducing more enzyme flexibility cannot result in a more enantioselective enzyme. In both studies, the same enzymes and organic media were used but different reactions were investigated. In this communication, a rationale is presented to explain both results.

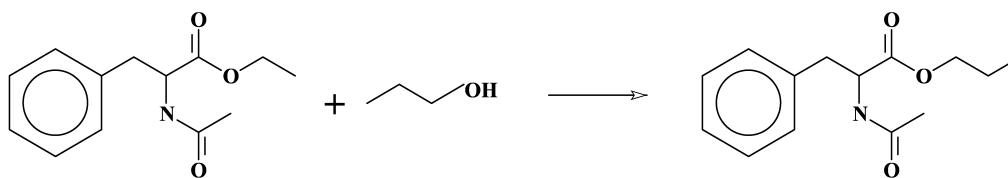
CONFLICTING EVIDENCE

Rariy and Klivanov studied the reaction between vinyl butyrate and racemic sec-phenylethyl alcohol, catalyzed by subtilisin Carlsberg or α -chymotrypsin in various organic solvents (reaction 1). All reactions were studied at a fixed water activity (a_w) and the effect of introducing extra water on the

*Tel.: +31-50-3634277. Fax: +31-50-3634165. E-mail: j.broos@chem.rug.nl



reaction 1



reaction 2

enantioselectivity (E) was determined. Possibly as a result of the fixed a_w , the earlier reported relationship, that an increase in $\log P$ of the solvent correlates with an increase in enzyme enantioselectivity for this reaction, was not observed (Fitzpatrick and Klibanov, 1991). In all nine organic solvents investigated, introduction of extra water resulted in the same or up to 2.6 times lower enantioselectivity. Except for the result in acetonitrile,[†] these results are in agreement with earlier studies in which the effect of water or water mimics on this reaction was addressed (Fitzpatrick and Klibanov, 1991; Santos *et al.*, 1999). Apparently, hydration of dry subtilisin Carlsberg or α -chymotrypsin, inducing higher enzyme flexibility (Broos *et al.*, 1995c), results in a relaxed enzyme enantioselectivity. This relationship is opposite to the relationship between enzyme enantioselectivity and enzyme flexibility found by Broos *et al.* for the same enzyme catalyzing reaction 2, the transesterification of N -acetyl-amino acid esters by 1-propanol (Broos *et al.*, 1995c). These substrates have been extensively used to study the mechanism of serine proteases under aqueous and non-aqueous conditions (Bender and Kedzy, 1965; Koskinen and Klibanov, 1996; Fersht, 1999). In 1988 the Klibanov group reported that the very high enantioselectivity of serine proteases towards N -acetyl-amino acid esters in water is dramatically relaxed when these enzymes are suspended in dry organic solvents (Sakurai *et al.*, 1988). For subtilisin Carlsberg, a linear relationship was observed between the enzyme enantioselectivity and the $\log P$ of the solvent, e.g. the enzyme enantioselectivity was the highest in polar solvents like DMF and acetonitrile and the lowest in apolar solvents like

dibutyl ether and cyclohexane. This result was rationalized with a "simple model" proposing that the displacement of water molecules from the active site upon binding of the good fitting L enantiomer becomes less favorable when the $\log P$ of the solvent increases. Because this effect is less pronounced for the sloppy fitting D enantiomer, the enantioselectivity was predicted to drop if the $\log P$ of the solvent increases. In a later contribution (Tawaki and Klibanov, 1992), the partitioning of the substrate between solvent and active site, which is different for the two enantiomers when bound in productive mode, was used to rationalize the relationship between enzyme enantioselectivity and $\log P$ for this reaction.

We have investigated the enantioselectivity of subtilisin Carlsberg, suspended in organic media towards N -acetyl-D,L-amino acid alkyl esters at fixed a_w conditions (Broos *et al.*, 1995a,b,c). The same relationship between enzyme enantioselectivity and $\log P$, as reported by Sakurai *et al.*, was observed. The flexibility of the enzyme under these reaction conditions was probed by monitoring the rotational mobility of a dansyl group in the active site, using the time-resolved fluorescence anisotropy technique (Broos *et al.*, 1995c). Five solvent systems were investigated including the most polar and apolar solvents used by Sakurai *et al.* (DMF and cyclohexane, respectively). A correlation between enzyme flexibility and enantioselectivity was found, e.g. the enzyme becomes more enantioselective if the solvent system allows the enzyme more flexibility. Although the same enzyme and solvent systems were used, this enzyme enantioselectivity versus enzyme flexibility relationship is opposite to the behavior of

[†]For subtilisin Carlsberg in acetonitrile however, a significant increase in enzyme enantioselectivity was found by the same group when 0.4% extra water was introduced (Fitzpatrick and Klibanov, 1991).

subtilisin Carlsberg in reaction 1. Rariy and Klivanov (2000), citing our work, claim that although subtilisin Carlsberg is more flexible in those solvents in which it has a higher enantioselectivity, this relationship is not causal. By referring to the classical "two site" enantioselectivity model they suggest that only a decrease in enzyme flexibility can result in a higher enantioselectivity. Below several arguments are presented showing that in catalyzing reaction 2 serine proteases need an enzyme flexibility approaching the situation in water in order to exhibit a high enantioselectivity.

A UNIFYING RATIONALE

In reaction 2 specific substrates, which closely resemble the structure of the natural substrates of these enzymes, e.g. peptides built up of L amino acids, are involved. In the case of a racemic mixture, the *N*-acetyl-L-amino acid ester is the specific substrate while the D enantiomer must be classified as non-specific (Jones and Beck, 1976). The more specific the L substrate is, the higher the enzymatic rate enhancement is compared to the uncatalyzed reaction (higher $(k_{\text{cat}}/K_{\text{M}})_{\text{cat}}/(k_{\text{cat}}/K_{\text{M}})_{\text{uncat}}$ ratio[†]). In terms of transition state theory the more specific the substrate, the better the enzyme is able to use binding energy of the enzyme-substrate complex to lower the free energy of the transition state (Fersht, 1999). The high rate enhancement towards the L substrate is only found under optimized reaction conditions, an aqueous buffer at optimal pH, temperature and ionic strength. Modeling studies show that conformational changes in both the enzyme and the substrate structure at the active site are essential for high enzymatic activity (Bruice and Benkovic, 2000). The restricted enzyme flexibility found when the enzyme is suspended in dry organic media is known to correlate with the much lower enzyme activity under these conditions and this is well documented for L substrates in reaction 2 (Koskinen and Klivanov, 1996). Because no specific interactions between the D substrate and the enzyme can be generated, a very low enzymatic rate enhancement is found, both under optimal aqueous conditions (for the L substrate) and under dry non-aqueous conditions. An increase in enzyme flexibility is thus expected to increase predominantly the activity towards the L substrate, resulting in a higher enzyme enantioselectivity. A modeling study about the enantioselectivity of α -chymotrypsin confirmed that only enantioselectivity was observed if the enzyme structure was allowed to be flexible (Wipff *et al.*, 1983). $k_{\text{cat}}/K_{\text{M}}$ values for serine proteases towards both the L and D amino acid substrates

TABLE I Activity of Subtilisin Carlsberg towards both enantiomers of *N*-acetyl-phenylalanine alkyl ester in cyclohexane ($a_{\text{w}} = 0.017$) and in aqueous buffer and the corresponding enantioselectivity (*E*)

	$k_{\text{cat}}/K_{\text{M}}$ (L) [$\text{M}^{-1}\text{s}^{-1}$]	$K_{\text{cat}}/K_{\text{M}}$ (D) [$\text{M}^{-1}\text{s}^{-1}$]	<i>E</i>
Cyclohexane*	0.39	0.021	18
Aqueous buffer†	54000	0.45	1.2 E5

*Data of ethyl ester from Broos *et al.* (1995b). The *E* value and the $k_{\text{cat}}/K_{\text{M}}$ values for the L and D enantiomers have been calculated using a racemic substrate mixture. †Data of methyl ester from Polgár and Fejes (1979).

under aqueous conditions are scarce in the literature because of the difficulty in measuring the very low activity towards the D substrate. For *N*-acetyl-phenylalanine alkyl esters these values have been reported for both enantiomers under aqueous and non-aqueous conditions (Table I). Formally, because of the effect of solvent on the thermodynamic reactant activity, the $k_{\text{cat}}/K_{\text{M}}$ values cannot be directly compared (Wagikar *et al.*, 1993; Wolff *et al.*, 1997; Halling, 2000). However, the trend is obvious, the very low activity of subtilisin Carlsberg in dry organic solvents is due to an enormous decrease of the activity towards the L enantiomer. Introduction of extra water and water mimics into the dry organic medium have been reported to both increase the activity towards the L enantiomer and result in a higher enzyme enantioselectivity (Ståhl *et al.*, 1991; Kawashiro *et al.*, 1995; Broos *et al.*, 1995a,b,c; Kawashiro *et al.*, 1996). Bringing the enzyme in a more natural environment, e.g. increasing the enzyme flexibility towards a level the enzyme displays in aqueous buffer, the enormous catalytic power for the specific L substrate is recovered, resulting in an observed increase in enzyme enantioselectivity. Clearly, the relationship between enzyme flexibility and enantioselectivity is causal for this reaction and this relationship is very useful in optimizing the enzyme enantioselectivity under non-aqueous conditions towards specific substrates. The reason why the enzyme is more flexible in polar solvents rather than in apolar solvents at a certain a_{w} is not clearly understood yet. A decrease in ordering of enzyme-bound water has been postulated to account for the fact that enzymes become more flexible when the solvent polarity increases (Nurok *et al.*, 1999) and more research is needed to elucidate this relationship.

For optimization of the enzyme enantioselectivity towards non-specific substrates a different strategy is needed. Here, the discrimination between enantiomers is merely governed by the ability of the enantiomers to approach the active site, e.g. the protein acts as a chiral surface where the chemical modification takes place. Because the potential to generate specific binding energy between enzyme

[†] $(k_{\text{cat}}/K_{\text{M}})_{\text{uncat}}$ signifies the second order rate constant of the uncatalysed reaction.

and substrate during the transition state is limited, low $(k_{\text{cat}}/K_{\text{M}})_{\text{cat}}/(k_{\text{cat}}/K_{\text{M}})_{\text{uncat}}$ ratios are found. Under these conditions a rigid enzyme might indeed exhibit the largest enantioselectivity since a rigid conformation can allow only one enantiomer to properly enter the active site. Rariy and Klibanov (2000) use this argument to explain their results. Because the catalytic potential of enzymes towards non-specific substrates is limited, variations in reaction medium can have a relatively large impact on the non-covalent substrate-enzyme interactions governing the enantioselectivity. This might be the reason that introduction of extra water can also significantly increase the enantioselectivity (Kitaguchi *et al.*, 1990; Högberg *et al.*, 1993) and complicates the optimization of the enzyme enantioselectivity for non-specific substrates.

The relaxed enantioselectivity of serine proteases towards amino acid substrates in dry organic media has been proposed to be useful for the enzymatic transformation of D substrates (Margolin *et al.*, 1987). However, because the relaxed enantioselectivity is due to a much lower activity towards the L enantiomer while the activity towards the D enantiomer is of the same order of magnitude (Table I), this argument is of limited use. Indeed, very long reaction times have been reported in the transformation of D substrates by serine proteases in organic media (Margolin *et al.*, 1987). What is needed is a methodology to activate the enzyme under non-aqueous conditions towards D substrates. Pretreatment of the enzymes with crown ether has been demonstrated to be effective in this; in a dry organic medium 18-crown-6 pretreated α -chymotrypsin exhibits a 65 fold higher $k_{\text{cat}}/K_{\text{M}}$ value toward a *N*-acetyl-D-amino acid ester than the $k_{\text{cat}}/K_{\text{M}}$ value determined in aqueous solution at optimal pH (Broos *et al.*, 1995b).

The large impact of enzyme flexibility on the activity towards specific substrates is also expected to affect the changed substrate specificity in organic media compared to aqueous buffer. While phenylalanine esters are much better substrates for α -chymotrypsin and subtilisin Carlsberg than serine esters in aqueous buffer, inverted substrate specificity was found in octane (Zaks and Klibanov, 1986) and various other organic solvents (Wescott and Klibanov, 1993). These results were explained in terms of "differential solvation". As observed in the case of the enantioselectivity, an increase in enzyme flexibility will especially affect the activity towards the more specific phenylalanine substrate compared to the less specific serine substrate. Investigation of the above reactions at several a_{w} values can elucidate the effect of enzyme flexibility on the substrate specificity and will show if both enzyme flexibility and differential solvation must be taken into account to explain the observed substrate specificity.

In conclusion, increase in enzyme flexibility is expected to result in a higher enzyme enantioselectivity towards racemic mixtures in which one of the enantiomers is a specific substrate for the enzyme. Towards non-specific substrates the predictive power of this relationship is limited and both higher and lower enantioselectivities have been observed if the enzyme flexibility is increased. The argument of Rariy and Klibanov (2000) that the enzyme enantioselectivity of subtilisin Carlsberg correlates perfectly with the log P value of the solvent is correct for reaction 2 (Sakurai *et al.*, 1988; Broos *et al.*, 1995c) but is in conflict with their own results about reaction 1. When enzyme flexibility and the distinction between specific and non-specific substrates is taken into account, the enantioselectivity of both reaction 1 and 2 can be properly explained.

References

- Bender, M.L. and Kedzy, F.J. (1965) "Mechanism of action of proteolytic enzymes", *Annu. Rev. Biochem.* **34**, 49–76.
- Broos, J., Engbersen, J.F.J., Verboom, W. and Reinhoudt, D.N. (1995a) "Activity and enantioselectivity of serine proteases in transesterification reactions in organic media", *J. Chem. Soc. Perkin Trans. 1*, 2899–2905.
- Broos, J., Engbersen, J.F.J., Verboom, W. and Reinhoudt, D.N. (1995b) "Inversion of enantioselectivity of serine proteases", *Recl. Trav. Chim. Pays-Bas* **114**, 255–257.
- Broos, J., Visser, A.J.W.G., Engbersen, J.F.J., van Hoek, A. and Reinhoudt, D.N. (1995c) "Flexibility of enzymes suspended in organic solvents probed by time-resolved fluorescence anisotropy. Evidence that enzyme activity and enantioselectivity are directly related to enzyme flexibility", *J. Am. Chem. Soc.* **117**, 12657–12663.
- Bruice, T.C. and Benkovic, S.J. (2000) "Chemical basis for enzyme catalysis", *Biochemistry* **39**, 6267–6274.
- Carrea, C., Ottolina, G. and Riva, S. (1995) "Role of solvents in the control of enzyme selectivity in organic media", *Trends Biotechnol.* **13**, 63–70.
- Fersht, A. (1999) *Structure and Mechanism in Protein Science*, 3rd Ed. (W.H. Freeman, New York).
- Fitzpatrick, P.A. and Klibanov, A.M. (1991) "How can the solvent affect enzyme enantioselectivity?", *J. Am. Chem. Soc.* **113**, 3166–3171.
- Halling, P.J. (2000) "Biocatalysis in low-water media: understanding effects of reaction conditions", *Curr. Opin. Chem. Biol.* **4**, 74–80.
- Högberg, H.-E., Edlund, H., Berglund, P. and Hedenström, E. (1993) "Water activity influences enantioselectivity in a lipase-catalyzed resolution by esterification in an organic solvent", *Tetrahedron Asymmetry* **4**, 2123–2126.
- Jones, J.B. and Beck, J.F. (1976) "Asymmetric syntheses and resolution using enzymes", In: Jones, J.B., Sih, C.J. and Perlman, D., eds, *Applications of Biochemical Systems in Organic Chemistry*. Part 1 (Wiley, New York), p 119.
- Kawashiro, K., Sugahara, H., Sugiyama, S. and Hayashi, H. (1995) "Effects of water content on the enantioselectivity of chymotrypsin catalysis in organic media", *Biotech. Lett.* **17**, 1161–1166.
- Kawashiro, K., Sugahara, H., Tsukioka, T., Sugiyama, S. and Hayashi, H. (1996) "Effect of ester moiety of substrates on enantioselectivity of protease catalysis in organic media", *Biotech. Lett.* **18**, 1381–1386.
- Kitaguchi, H., Itoh, I. and Ono, M. (1990) "Effects of water and water-mimicking solvents on the lipase-catalyzed esterification in an apolar solvent", *Chem. Lett.*, 1203–1206.
- Koskinen, A.M.P., Klibanov, A.M., (1996) *Enzymatic Reactions in Organic Media* (Blackie, London).

- Margolin, A.L., Tai, D.-F. and Klibanov, A.M. (1987) "Incorporation of D-amino acids into peptides via enzymatic condensation in organic solvents", *J. Am. Chem. Soc.* **109**, 7885–7887.
- Nurok, D., Kleyale, R.M., Muhoberac, B.B., Frost, M.C., Hajdu, P., Robertson, D.H., Kamat, S.V. and Russell, A.J. (1999) "Study of enzyme-catalyzed reactions in organic solvents using multiple linear regression", *J. Mol. Catal. B Enzym.* **7**, 273–282.
- Polgár, L. and Fejes, J. (1979) "Mechanism-controlled stereospecificity", *Eur. J. Biochem.* **102**, 531–536.
- Rariy, R.V. and Klibanov, A.M. (2000) "On the relationship between enzymatic enantioselectivity in organic solvents and enzyme flexibility", *Biocat. Biotrans.* **18**, 401–407.
- Rupley, J.A. and Careri, G. (1991) "Protein hydration and function", *Adv. Protein Chem.* **41**, 37–172.
- Sakurai, T., Margolin, A.L., Russell, A.J. and Klibanov, A.M. (1988) "Control of enzyme enantioselectivity by the reaction medium", *J. Am. Chem. Soc.* **110**, 7236–7237.
- Santos, A.M., Clemente, I.M., Barletta, G. and Griebenow, K. (1999) "Activation of serine protease subtilisin Carlsberg in organic solvents: combined effect of methyl-beta-cyclodextrin and water", *Biotech. Lett.* **21**, 1113–1118.
- Stahl, M., Jeppsson-Wistrand, U., Månsson, M.-O. and Mosbach, K. (1991) "Induced stereoselectivity and substrate selectivity of bio-imprinted chymotrypsin in anhydrous organic media", *J. Am. Chem. Soc.* **113**, 9366–9368.
- Tawaki, S. and Klibanov, A.M. (1992) "Inversion of enzyme enantioselectivity mediated by the solvent", *J. Am. Chem. Soc.* **114**, 1882–1884.
- Wagikar, P.P., Graycar, T.P., Estell, D.A., Clark, D.S. and Dordick, J.S. (1993) "Protein and solvent engineering of subtilisin BPN' in nearly anhydrous organic media", *J. Am. Chem. Soc.* **115**, 12231–12237.
- Wescott, C.R. and Klibanov, A.M. (1993) "Solvent variation inverts substrate specificity of an enzyme", *J. Am. Chem. Soc.* **115**, 1629–1631.
- Wipff, G., Dearing, A., Weiner, P.K., Blaney, J.M. and Kollman, P.A. (1983) "Molecular mechanics studies of enzyme-substrate interactions: The interaction of L- and D-N-acetyltryptophanamide with α -chymotrypsin", *J. Am. Chem. Soc.* **105**, 997–1005.
- Wolff, A., Straathof, A.J.J., Jongejan, J.A. and Heijnen, J.J. (1997) "Solvent induced change of enzyme enantioselectivity: rule or exception?", *Biocat. Biotrans.* **15**, 175–184.
- Zaks, A. and Klibanov, A.M. (1986) "Substrate specificity of enzymes in organic solvents vs. water is reversed", *J. Am. Chem. Soc.* **108**, 2767–2768.